A sticky situation: Aberrant protein–protein interactions in Parkinson's disease

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ABSTRACT:

The aberrant aggregation of normally soluble proteins into amyloid fibrils is the pathological hallmark of several neurodegenerative disorders, including Alzheimer's and Parkinson's diseases. Understanding this process will be key to developing both diagnostic and therapeutic approaches for neurodegenerative diseases. Recent advances in biophysical techniques, coupled with kinetic analyses have enabled a thorough description of the key molecular steps involved in protein aggregation. In this review, we discuss these advances and how they have been applied to study the ability of one such protein, α-Synuclein, to form neurotoxic oligomers.

1) Introduction

While protein oligomerisation and polymerisation is crucial for many biological processes [1–7], the aberrant self-association and aggregation of some proteins has been implicated in a range of diseases. Amyloid fibrils, in particular, have been associated with an increasing number of disorders, including among others, type-II diabetes [8], neurodegenerative diseases [9–11] and cancer [12].

Amyloid deposits in these diseases are generally composed of, primarily, a single disease-specific protein, for instance α-Synuclein (αS) positive Lewy bodies in Parkinson's disease (PD) [9], and extracellular amyloid beta (Aβ) plaques [10] and intracellular Tau tangles [11] in Alzheimer's disease (AD). A range of other molecules, however, are also associated with deposits *in vivo*, and these tend to include molecular chaperones and other proteins involved in cellular quality control processes [13,14].

The amyloid structure is a highly thermodynamically stable state, and the ability to form amyloid appears to be a generic feature of the polypeptide backbone of proteins [15–17]. Indeed, polypeptides composed of only one type of amino acid are able to form well-

defined fibrils [16]. Despite this, the propensity of a protein to form amyloid fibrils depends greatly on its amino acid composition, with side chains being crucial in regulating both the kinetic barriers to amyloid formation and the lateral packing of β-sheets [18–20]. The native state sequesters aggregation-prone regions in the core of the protein to prevent intermolecular contacts from forming, and the cooperativity of the folding process also plays a role in forming kinetic barriers against aggregation [21,22]. Aggregation-prone residues or sequences are evolutionarily selected against in exposed protein regions, and highly aggregation-resistant residues (gatekeeper residues) are often found close to exposed aggregation prone regions [23,24].

Unlike native monomeric protein, the relative stability of the amyloid structure is dependent on protein concentration [25], and this leads to the concept of a critical concentration above which the most stable state is amyloid. Surprisingly, many amyloidogenic proteins are expressed *in vivo* above this concentration [25]. So for at least some proteins, including αS, β-2-microglobulin and αB-crystallin, the native state of the protein is metastable with respect to the amyloid state [25]. It is, however, a rare event for proteins to form such structures *in vivo* due to high kinetic barriers, and the presence of inhibitory factors such as molecular chaperones.

The protein aggregation process is complex, involving many different intermediates, and a range of pathways, some of which may be on route to the formation of amyloid fibrils, and others which may form off-pathway, but potentially toxic species. For this reason, novel techniques and advanced analytical methodologies have been developed to study protein aggregation. This review discusses these with a focus on αS, the protein associated with neurodegenerative disorders, such as PD.

2) α-Synuclein structure and function

αS is a 140 residue protein expressed at high levels (40-45 μM [26]) in neurons, and under physiological conditions is mainly found in pre-synaptic neuronal terminals, near synaptic vesicles [26]. Misfolding and aberrant accumulation of αS has been implicated in a range of neurodegenerative diseases termed the synucleinopathies, most notably PD, but also several other diseases such as dementia with Lewy bodies and multiple system atrophy [27].

The αS amino acid sequence consists of three major regions: the amino-terminal (N-terminal) region, the hydrophobic non-amyloid beta component (NAC) region, and the acidic carboxy-terminal (C-terminal) region (*Figure 1*). The N-terminal region contains four imperfect repeats of the motif KTKEGV which are important in lipid membrane interactions. The central hydrophobic NAC section is so termed due to its identification in the extracellular plaques of AD brains [28]. The C-terminal region of the protein is highly acidic, containing ten glutamate and aspartate residues. It is responsible for the high thermostability of αS. It has also been shown to regulate the protein's aggregation, since C-terminally truncated fragments aggregate faster than the full length protein,[29–

31] and post-translational modifications, such as phosphorylation, of C-terminal residues modulate the aggregation propensity of αS [32].

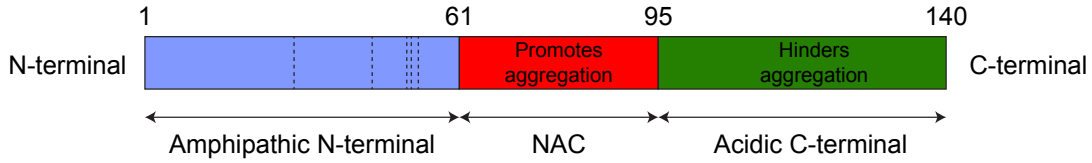

Figure 1. Primary structure of αS. The structure is divided into three regions; the N-terminal amphipathic repeat region; the central NAC region, which contains most of the hydrophobic residues, promoting aggregation; and the acidic C-terminal region, which hinders protein aggregation. Dashed lines show the A30P, E46K, H50Q, G51D and A53T mutations, which are genetically linked to Familial PD.

αS is a member of the intrinsically disordered protein (IDP) class, as it has little or no static structure under physiological conditions. Instead, it has been shown to switch between an ensemble of dynamically interchanging conformations [33]. Sandal *et al.* have used atomic force microscopy (AFM) to probe these conformations at the single-molecule level [34], finding that it occupies three distinct classes: random coil (populated 38% of the time), β-like structures (populated 7.3% of the time) and those stabilised by short and long-range mechanically weak interactions (54.5% of the time). Some of these conformations are stabilised upon binding to other proteins or to lipid membranes; for example, a number of studies have shown that αS adopts an α-helical conformation when bound to natural and synthetic phospholipid membranes [35–37].

Bartels [38] and Wang [39] have suggested that αS may actually remain helical in its native environment, existing in a multimeric state. They argue that the denaturing conditions used to purify recombinantly expressed αS may account for its unfolded state. Through a combination of cross-linking and analytical ultracentrifugation of αS extracted from brain and red blood cells, Bartels identified the protein as a folded, helical tetramer. Wang used Electron Microscopy reconstruction and NMR to study αS purified under non-denaturing conditions, and also identified a helical multimeric state that resisted aggregation. Fauvet *et al.*, however, have purified recombinant αS under non-denaturing conditions and have concluded that it is still an IDP [40], and Burre *et al.* showed that native αS extracted from brain was purely monomeric [41]. In-cell NMR and EPR experiments have revealed αS to be predominantly a disordered monomer in a number of neuronal and non-neuronal mammalian cell-lines [42].

The lack of a distinct secondary or tertiary structure in IDPs allows them to fulfil a key role in molecular recognition of their partner proteins. There are several advantages of structural disorder, such as the ability to bind strongly with low specificity, an increased rate of interaction, and effective regulation by degradation. The specific role of αS is still unclear; however it has been reported to be involved in the storage, transmission and biosynthesis of dopamine [43], protein trafficking and stabilisation of synaptic vesicles [44], and to play a role in mitochondrial function and neuronal protection [45]. Despite not knowing its specific role, αS knockout and knockdown mice display altered

neurotransmission [46], decreased dopamine levels [47], and also died prematurely [48].

3) α-Synuclein and Parkinson's Disease

PD is a sporadic neurodegenerative disorder resulting from the progressive loss of dopaminergic neurons in the *substantia nigra*, a region of the midbrain involved in the control of movement and coordination. Symptoms include muscular rigidity, resting tremor and bradikinesia (slowness in the initiation of movement) [49]. It is the second most common neurodegenerative disease, and the most common movement disorder with the primary risk factor for the disease being age. Levodopa, a dopamine precursor molecule, is used to treat the motor symptoms of patients, but can exacerbate cognitive and neuropsychiatric symptoms [50,51]. There is currently no therapy to treat the underlying cause of the disease.

The formation of cytoplasmic inclusions termed Lewy bodies in surviving neurons is the main pathological feature of the disease. Lewy bodies, and Lewy neurites (abnormal neurites that contain Lewy body-like filaments), are formed mainly from αS, which aggregates into amyloid fibrils [9,17,52–54]. These inclusions also contain many other components such as ubiquitin, synphilin-1 and phosphorylated neurofilaments [55,56]. Indeed over 90 components of Lewy bodies have been identified by immunohistochemical methods [57] (*Figure 2*), and several hundred by mass-spectrometry based proteomic methods [58,59]. As well as containing a number of proteins with a high intrinsic aggregation propensity [60,61], these also include many components of the proteostasis network such as molecular chaperones, proteins involved in oxidative stress response, aggresome-related proteins, the autophagy-lysosomal pathway and members of the ubiquitin-proteasome system [57] (see *Figure 2*). These proteins have been shown to influence key steps in the αS aggregation pathway [62,63], and depletion of these components by their sequestration into insoluble aggregates can have large effects on cellular toxicity and disease progression [64].

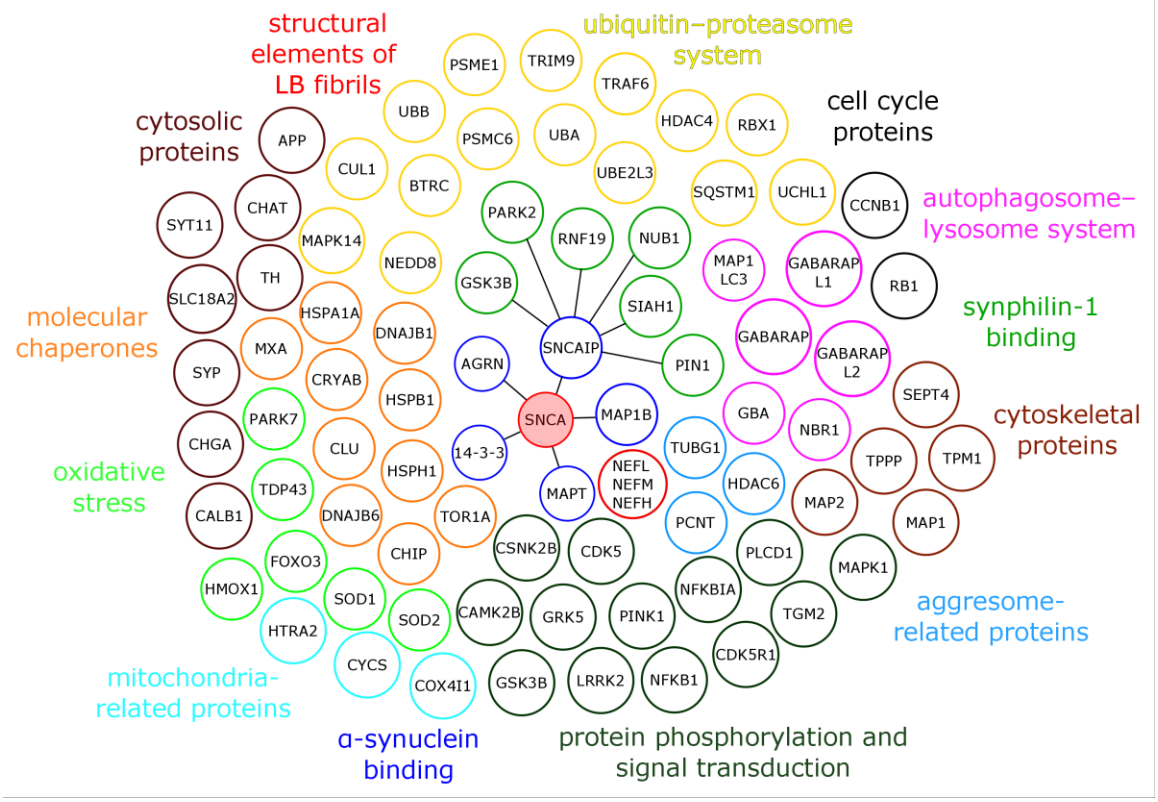

Figure 2 Lewy body components grouped by broad functional categories [57]*.These include proteins with a functional role in preventing pathological protein aggregation in neurodegenerative disease (e.g. HSPB1* [65]*, CRYAB* [66]*) and proteins which themselves have been implicated in the pathology of protein misfolding diseases (e.g. TDP43* [67]*, SOD1* [68]*, APP* [69]*)*

Mis-sense point-mutations (A30P [70], E46K [71], H50Q [72], G51D [73], A53E [74] and A53T [75]) in αS lead to a rare genetically inherited form of early-onset PD. The phenotype is inherited in an autosomal dominant manner in families carrying any of these mutations. A triplication of the wild-type αS gene also causes early-onset PD [76]. These mutations affect the aggregation propensity of αS leading to amyloid formation *in vitro*, and the fibrils formed appear identical in atomic force microscopy (AFM) analysis [77,78].

In both familial and sporadic PD, certain post-translational modifications of αS are observed at greatly increased levels, including phosphorylation at residues Ser87 [79], Tyr125 [80] and Ser129 [32] and nitration at residues Tyr39, Tyr125, Tyr133 and Tyr136 [81]. In particular, phosphorylation at Ser129 is found in nearly 90% of deposited αS, as compared to around 4% in healthy controls [32,82]. In addition, C-terminal truncations of αS are found in relatively high concentrations in Lewy bodies and have been shown to have an increased aggregation propensity *in vitro* [83]. Interestingly, they are also capable of co-aggregation with the full-length protein, thereby promoting the aggregation process [83].

4) Protein aggregation

a) Methods to study protein aggregation

The formation of amyloid progresses through a number of precursor species, including monomeric unfolded or partially unfolded proteins. These then associate to form soluble oligomeric intermediates and protofibrillar structures, which compose the mature fibril [84]. In particular, soluble oligomers on the pathway to amyloid formation are now thought to be the major toxic species in classical amyloid diseases such as PD and AD [85,86]. These soluble aggregates progress to form protofilaments; short, thin, fibrillar species that assemble into mature fibrils.

i) Traditional biochemical techniques

For decades, small fluorescent probes which bind to amyloid fibrils have been used to monitor the time evolution of the aggregation of a wide range of amyloidogenic proteins [87–89]. Thioflavin T (ThT) is the most widely used of these probes, and the fluorescence intensity of ThT has been shown to be proportional to the mass concentration of fibrils formed in a number of systems [90,91] enabling quantitative analysis of aggregation kinetics to be performed. These assays have been used extensively to investigate the influence of intrinsic and extrinsic factors on αS aggregation [62,77,92–94]; however care must be taken to ensure that exogenous compounds do not bias the ThT assay [95]. Moreover, in certain systems the fluorescence intensity can be nonlinear at sub-stoichiometric ThT concentrations due to self-quenching, and may influence the lateral packing of fibrils [96], effects which need to be controlled for in experimental design and analysis.

Bulk fluorescence experiments exhibit a lag-phase for the formation of amyloid fibrils, often thought of as a "waiting time" for nuclei to form. It is now clear that millions of primary nuclei are formed during the lag phase [97], which then grow (and under certain conditions proliferate) until a detectable aggregate concentration is reached. More recently, fluorescent probes with a higher sensitivity for oligomeric forms of αS have been developed [98], providing insights into early oligomer generation along the aggregation pathway.

Label-free bulk techniques include light scattering to monitor the formation of large particles over time, which can yield qualitative information on the formation of oligomeric species as well as fibrils, however for polydisperse samples data interpretation can be challenging [99]. Small angle x-ray scattering has also been used to monitor the evolution of oligomeric species over the time course of aggregation and provide some structural information [100].

ii) Surface-based methods

In order to overcome the difficulties of quantitation in bulk experiments, surface-based

methods have been developed to directly monitor aggregate growth. Quartz crystal microbalance (QCM) sensors have been used as a label-free technique to directly report on the change in mass of surface-attached fibrils and have enabled very precise measurements of the influence of solution conditions on αS fibril elongation to be obtained [101]. However, such techniques are sensitive to surface effects, for example, elongation may occur at a slower rate for fibrils attached to a surface.

iii) Single-molecule techniques

It is widely thought that the soluble oligomeric species that are formed on pathway to amyloid fibrils are the key cytotoxic species in many neurodegenerative diseases. However, studying such transient, and highly heterogeneous populations is challenging using traditional biophysical techniques, and for this reason, single-molecule approaches have been developed to characterize the soluble oligomers. By counting molecules one-by-one, such approaches are capable of directly measuring biomolecular behaviours and structures at equilibrium. Sub-populations of species are discretised, and analysed statistically, allowing insights into situations previously hidden by ensemble averaging.

Single-molecule confocal microscopy methods have been used to study a range of aggregating proteins, the first one being the SH3 domain of phosphatidylinositiol-3'-kinase (PI3-SH3) [102]. In this study, equimolar mixtures of two different dye-labelled PI3-SH3 solutions (Alexa Fluor 488 (AF488) and Alexa Fluor 647 (AF647)) were incubated under conditions favouring protein aggregation, during which time regular samples were taken and analysed using two color coincidence detection (TCCD). The TCCD technique relies on having confocal volumes of two different wavelengths overlapped, in this case 488 nm (blue) and 633 nm (red). As dye-tagged molecules diffuse through this volume, they generate a burst of fluorescence. Monomeric PI3-SH3 only gave rise to a signal in either the blue-excitation, or red-excitation channel, whereas the oligomers, which likely contained both dyes, generated a coincident signal in both channels. This method therefore provided the means to separate oligomeric events from the majority of monomeric events; the total intensity of the bursts could then be used to determine the approximate size of the oligomer.

The method was then adapted using single-molecule FRET to study the aggregation of αS (Figure 3) [85]. Rather than exciting with two wavelengths of light, the samples were only illuminated with 488 nm radiation, and photons emitted from both dyes were then detected. Only species with AF488 present were able to be excited; the monomers only emitting photons in the green channel, and the oligomers emitting photons in both the green and red channels (provided the AF488 and AF647 dyes were close enough for FRET to occur). In addition to the approximate size of the oligomers being determined, the methodology also allows the FRET efficiencies of the dye-labelled oligomers, which are indicative of structure, to be determined. At early time-points, the oligomers had low FRET efficiencies, were less cytotoxic, and were sensitive to proteinase-K digestion. Later oligomers had a higher FRET efficiency, were more cytotoxic, and had a higher resistance to proteinase-K digestion. This led to a new mechanism for early αS aggregation being proposed (Figure 3), in which the monomers firstly associate to form

non-toxic oligomers (nucleation), presumably lacking ß-sheet structure. The size of these oligomers could change, either via growth through addition of monomer, or dissociation through loss of monomer. Importantly, they could also undergo a structural reorganisation (half-life of approximately 30 hours) to form the cytotoxic, ß-sheet containing oligomers. Once again, these oligomers could also grow through addition of monomer, or decrease in size through loss of monomer.

This technique has been further advanced through the use of fast-flow microfluidics to increase the detection rate [103,104], allowing a higher time-resolution to be achieved. This has enabled precise kinetic analyses of the different steps in the αS aggregation to be determined, and has supported the notion that seeding alone cannot account for the spread of cytotoxicity, but that the spreading of cellular stress is also an important factor [105].

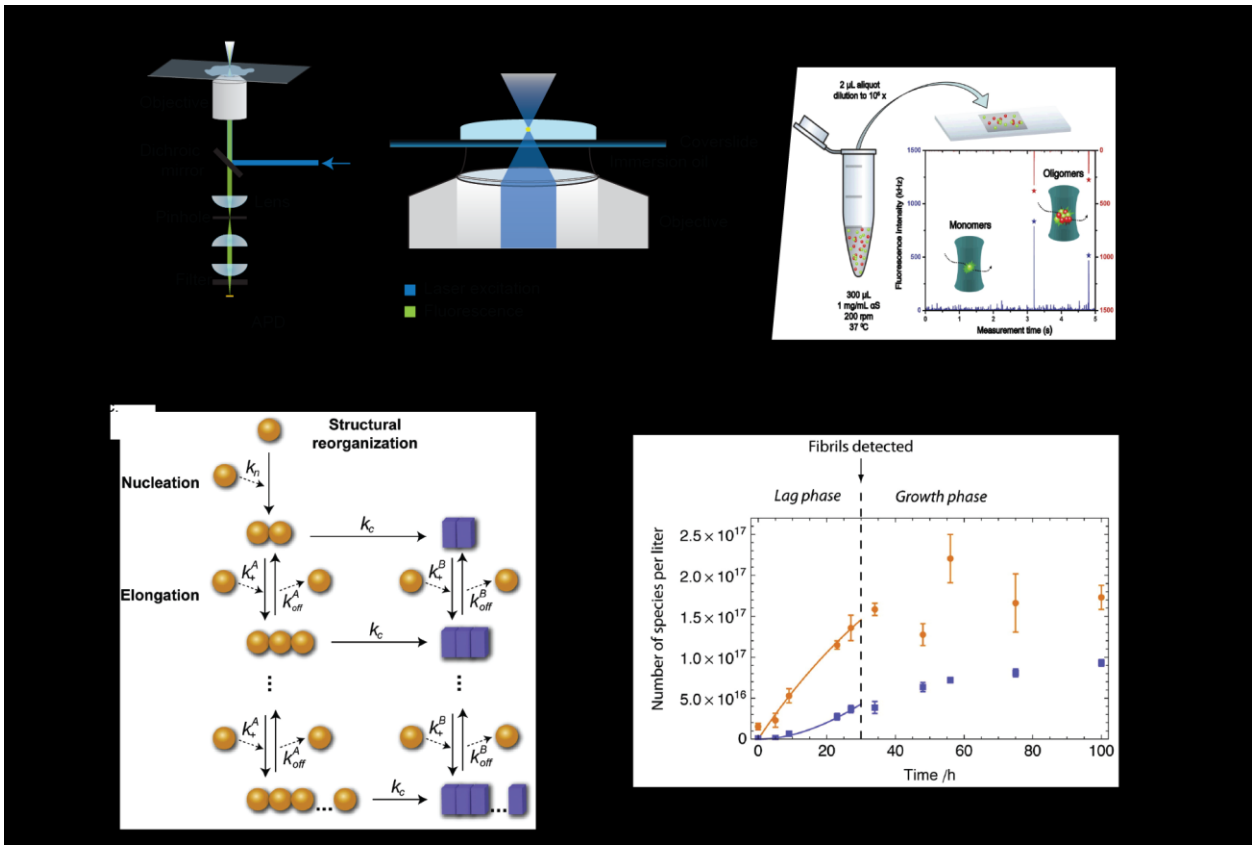

Figure 3. a. In confocal microscopy, the laser light is focused into a diffraction limited point, and molecules are excited as the pass through this probe volume. Fluorescence is detected using an Avalanche Photodiode Detector (APD). b. Schematic description of the experimental protocol for aggregation experiments. Bursts of fluorescence coincident in both channels indicate the presence of FRET-positive oligomeric species (marked as asterisks). Non-coincident bursts can be attributed to monomers and are normally much less bright than those corresponding to oligomers. c. Scheme for the minimalistic kinetic model used to fit the early stages of αS aggregation. d. Results of the global fitting

(continuous lines) of the kinetics of formation of the two types of oligomeric species estimated under bulk conditions from smFRET experiments. b, c and d adapted from [52].

Due to inhomogeneous excitation in confocal experiments, the accurate determination of size remains challenging. For example, a small oligomer passing through the center of the confocal volume may look just as bright as a larger oligomer containing lots of dye molecules passing through the periphery of the probe volume. This problem has been somewhat bypassed through the use of labelled αS and total internal reflection fluorescence microscopy (TIRFM). As individual dye molecules within the oligomers photobleach, there is a stepwise decrease in the total intensity of each individual spot. These steps can be counted to determine how many dye molecules were present in the oligomer; however, with too many dyes, this becomes challenging. By using a sub-stoichiometric labelled population of monomer, Zijlstra et al. [106] were able to identify a well-defined oligomer having 31 monomer units. However, this method relies upon the oligomers having a similar size and structure, and the one purified in this study may not be the most relevant to the disease.

In the above described methods, αS must be tagged with an organic fluorophore. In some cases the label may have an adverse effect on the behavior of the protein of interest [107]. Alternatively, dyes such as ThT, or the pentameric form of formyl thiophene acetic acid (pFTAA), can be coupled with TIRFM to directly detect protein aggregates. This has been used to quantify the elongation of individual αS fibrils, unveiling a “stop and go” mechanism of fibril elongation that was undetectable in ensemble measurements (Figure 4b) [108], a result also confirmed using *in situ* tapping mode atomic force microscopy [109]. Such methods have also been used to image individual aggregates in human cerebrospinal fluid [110], leading to the finding that samples from patients with PD contain more ThT-active species than those from healthy controls (Figure 4c,d). Although such methods remove the need for a label, they do have lower specificity. Which species are able to bind ThT is not fully understood, and it could be the case that some toxic oligomeric forms do not bind such dyes. Additionally, they dyes may also bind to other structures that are unrelated to neurodegeneration.

Hybrid methods, which combine cell-free expression with single-molecule techniques and brightness analysis, have enabled the oligomerisation and fibrillisation of αS disease-related mutants to be monitored in real-time without purification, denaturation or labeling steps [111].

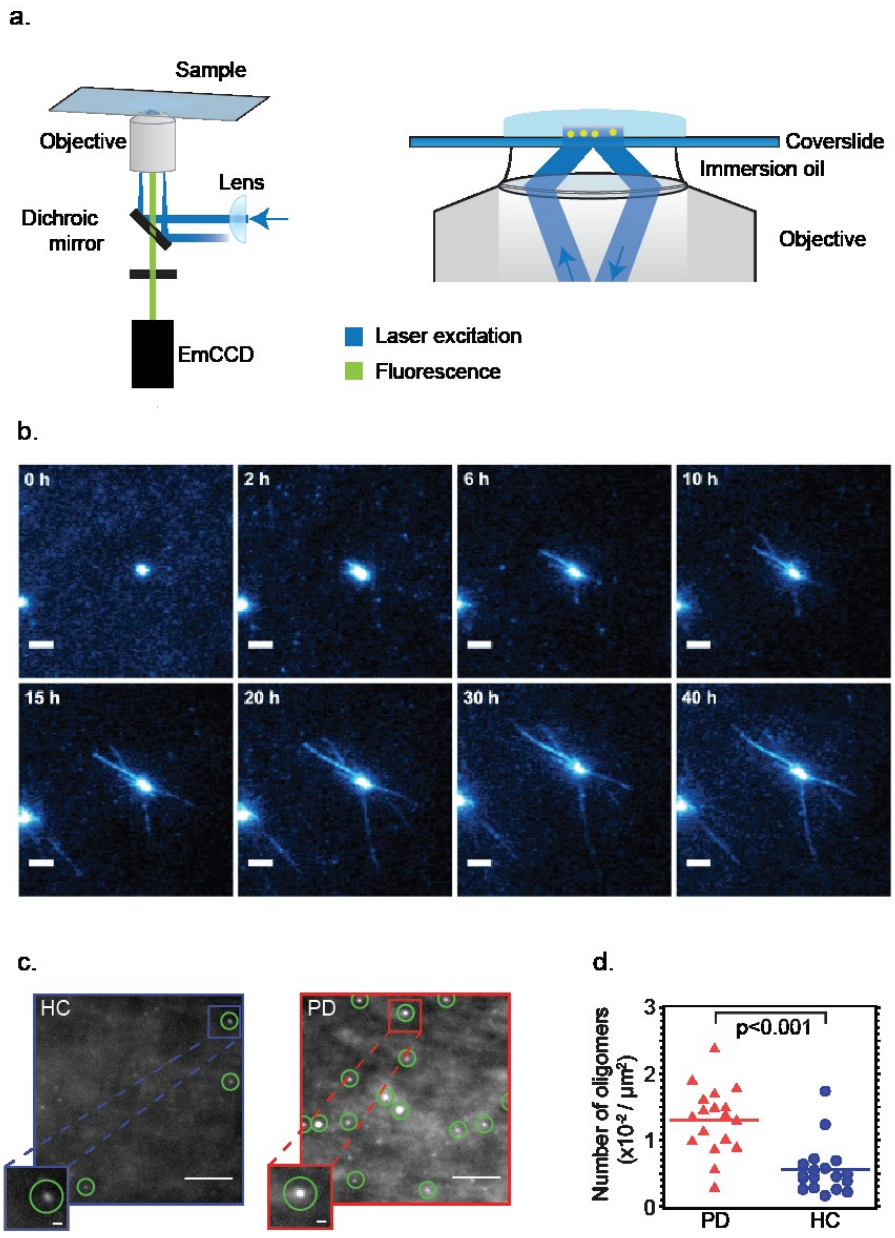

Figure 4 Detection of individual aggregates using TIRFM and ThT. a. TIRFM- laser light is totally internally reflected off the glass-water interface generating an evanescent wave that penetrates <200 nm into the sample. Fluorescence is detected using an electron multiplied CCD. b. Time series of TIRFM images of a solution (160 µM) of monomeric α-syn in the presence of 15 µg/ml seeds and 7 µM ThT. Excitation of ThT fluorescence with a 405-nm laser, filtering of fluorescence emission through a 450/50 bandpass filter. The scale bar represents 2 µm. Adapted from [108]. *c. Detection of individual aggregates in the CSF of HC and PD patients. Scale bar represents 5 µm and 500 nm in the zooms. d. Box plots of the same data for the number of oligomers detected. Horizontal lines show the mean counts for PD and HC samples. Adapted from* [110].

iv) Super-resolution techniques

Conventional optical methods are limited by the physical diffraction of light, having a resolution limit of approximately 250 nm. However, the advent of super-resolution microscopy methods [112–114] has improved the resolution of optical microscopy to enable objects as small as 5 nm [115] to be detected (for a comprehensive review see Horrocks et al [116]). Such methods have been used to characterize both the oligomers and fibrils formed from αS.

Two-color direct stochastic optical reconstruction microscopy (dSTORM) has revealed highly heterogenous elongation rates of individual fibrils, potentially due to fibril polymorphism or the spatial arrangement of fibril ends [117] (*Figure 5*a). It is possible that the dyes used may affect the fibril elongation process, and so the kinetic parameters determined may be different to those exhibited by the native protein.

A multi-dimensional super-resolution imaging technique referred to as spectrally-resolved PAINT (sPAINT) enabled the hydrophobicity of oligomers and fibrils to be mapped at the nanoscale (*Figure 5*b [118]). sPAINT makes use of the solvachromatic dye nile red, whose emission spectrum is red-shifted as its local environment becomes less hydrophobic. By measuring the wavelength of each excited dye molecule, in addition to its position at the nanoscale, the method has shown that αS oligomers have a higher surface hydrophobicity than fibrils, which could be related to their increased cytotoxicity.

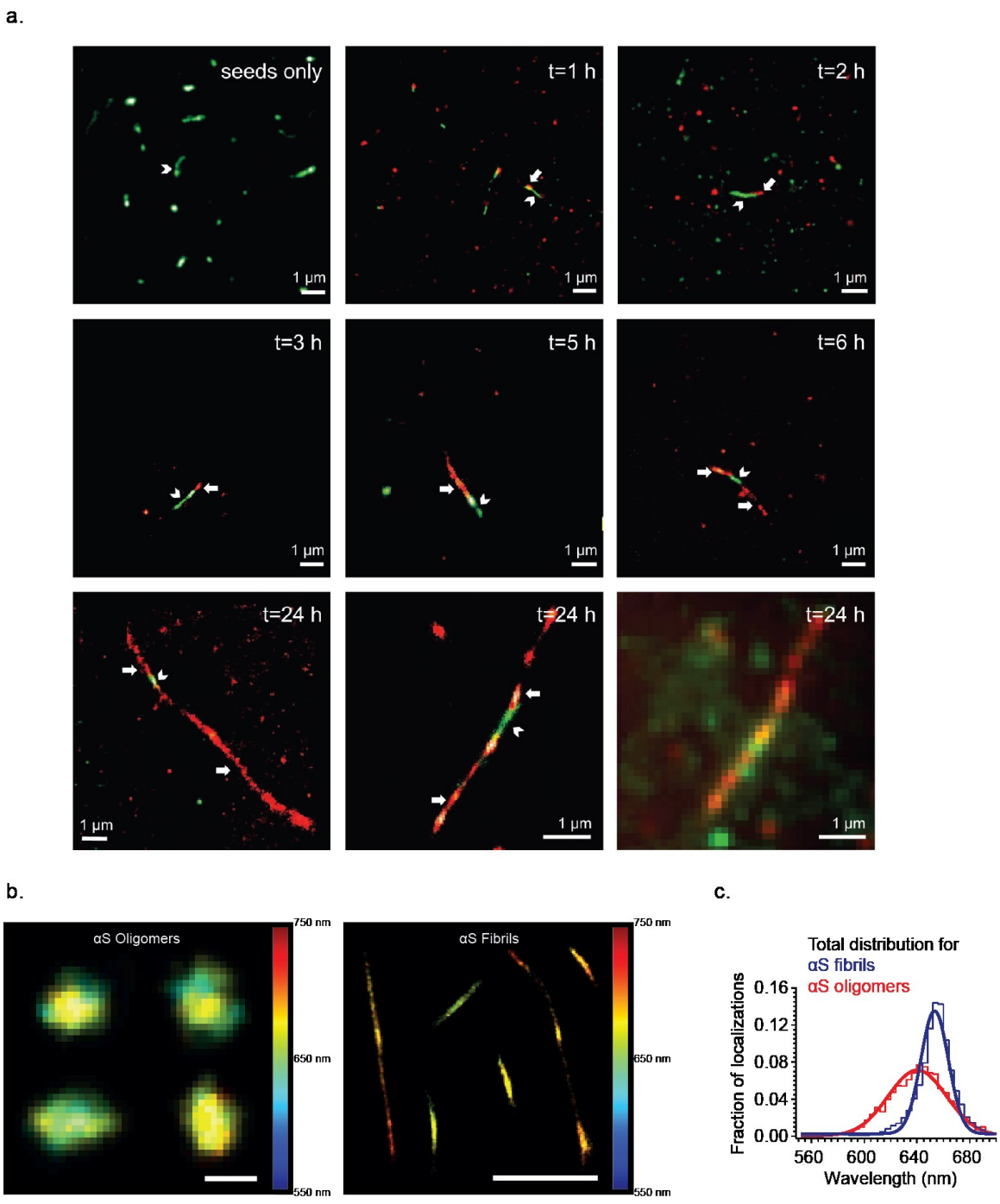

Figure 5 a. Two-color dSTORM images reveal details of the elongation of αS fibrils through addition of soluble protein molecules, in vitro. The initial sample consists of αS seed fibrils labelled with Alexa Fluor 568 (AF568) (green). At t = 0 h, monomeric αS labelled with AF647 (red) was added to the solution. Aliquots were taken during the aggregation reaction and imaged with two-color dSTORM. The images show the overlay of the two channels (green, AF568 and red, AF647). Each fibril consists of the initial seed (green, indicated by an arrowhead) and the extended region formed through addition of monomeric protein (red, indicated by an arrow). The lengths of the fibrils

reach several micrometers with the initial seed fibril being extended from both ends. The last image shows a conventional fluorescence image in TIRFM of the same fibril as in the previous box showing that the increase in resolution achieved by dSTORM enables the original and the newly formed section of the fibril to be clearly distinguished. Adapted from [117]. *b. Representative sPAINT hydrophobicity image of single αS oligomers (scale bar is 100 nm) and fibrils (scale bar is 1 μm). c. Total frequency histogram of the individual sPAINT localisations from both αS oligomers (red, n=17,619 localizations, N=539 oligomers; peak photon values above background ~780) and αS fibrils (blue, n=120,275 localizations, N=1,528 fibrils; peak photon values above background ~1,100) showing that the fibrils become less hydrophobic (red-shifted). b and c adapted from* [118].

b) Aggregation of αS in disease

The high expression level of αS in neurons is above experimentally determined values for the critical aggregation concentration, which range from 0.7-2.7 uM in solution [25,105] to low nanomolar in the presence of negatively charged lipid membranes [119]. αS is therefore an example of a metastable protein with respect to the amyloid state, with intrinsic kinetic barriers preventing self-assembly. These kinetic barriers are very high, and while the aggregation of αS into amyloid fibrils is a hallmark of PD, αS is incredibly stable in its monomeric form in solution. Under quiescent conditions, for example, no fibril formation is detected when concentrations of the monomeric protein (140 uM) is well above the critical aggregation concentration and incubated at 37 °C for 5 days [120]. Some form of experimental perturbation is therefore required to induce the aggregation of αS *in vitro* and a common technique is to shake the sample at 300-2200 r.p.m. which results in observable fibril-formation within hours [121,122]. Shaking increases both primary nucleation [122] and fibril fragmentation rates, with the fragmentation of fibrils particularly likely to be rate determining under these conditions [77,123].

Interestingly, under quiescent conditions, solution interfaces have been shown to induce αS fibril formation. In particular, the addition of components such as hydrophobic nanoparticles [124], or certain lipid membranes [120] promote primary nucleation by several orders of magnitude. These experiments have demonstrated that hydrophobic-hydrophilic interfaces have a strong influence on aggregate formation and indeed, under shaking conditions, removing the air-water interface from experimental samples dramatically decreases the aggregation rate of αS [125]. These observations have led to the development of experimental methods to investigate the initial steps in fibril formation by surface induced aggregation under quiescent conditions [77,120,126,127] allowing the influence of intrinsic and extrinsic factors on the primary nucleation of αS lipid-induced fibril formation to be determined.

The transition from soluble monomeric to fibrillar αS involves the population of multiple intermediate species, including oligomeric and protofibrillar structures, and αS fibrils themselves have been shown to populate different fibrillar strains [128,129]. These species have differential toxicities, with certain oligomeric states now appear to be the major toxic species [52,128,130,131]. There are several mechanisms for the formation of

oligomeric species (through both primary nucleation and secondary nucleation) which can interconvert between more or less toxic structural states [52,130]. The mechanism of toxicity has been proposed to be through the induction of cellular reactive oxygen species (ROS) production which stimulates apoptotic pathways in neuronal cells [132], and indeed oxidative stress has been linked to a range of neurodegenerative diseases [133]. Another proposed mechanism is via aberrant interactions with lipid membranes through exposed hydrophobic groups which cause the membranes to rupture [130]. The reduction in toxicity upon the formation of mature amyloid fibrils from these more toxic oligomeric precursors has been rationalised as being due to the reduction of exposed hydrophobic regions concurrent with a decrease in the surface to volume ratio [15,52,130].

Thus the conversion of αS monomers to the amyloid form can proceed through a variety of mechanisms, either via nucleation to form the soluble oligomeric precursor species, or from pre-existing aggregates acting as templates for the elongation of fibrils. Secondary processes including fibril fragmentation and surface-catalysed secondary nucleation (the formation of new soluble oligomeric species on the surface of pre-existing aggregates) lead to the rapid proliferation of aggregates, as they are autocatalytic growth processes, as well as an increase in the concentration of transient, and potentially toxic, oligomeric species.

c) Kinetics of protein aggregation

The toxicity associated with fibril-formation occurs to varying degrees through both a loss of function of the aggregated protein, and through the generation of toxic species in the process of amyloid formation. The major toxic species appear to be low molecular weight oligomeric intermediates, and targeted interventions to reduce their abundance along the aggregation pathway dramatically reduce the toxicity associated with protein aggregation [86]. Fibrils have been demonstrated to also be toxic, the injection of fibrils into mice expressing transgenic αS can seed the conversion of endogenous and expressed αS into neuronal inclusions which lead to selective degeneration of inclusion-containing neurons [134], and indeed structurally polymorphic fibrillar assemblies composed of the same amyloidogenic protein have been shown to have differential toxicities in cell culture [128] and animal models [135]. In the case of αS, different fibril strains have been proposed to be the cause of the varying pathologies seen in the synucleinopathies. In addition, since amyloid deposits *in vivo* contain a large number of components of the proteostasis system, the general increase in misfolded protein levels associated with proteostasis dysregulation is likely to exacerbate cellular toxicity [13]. In the case of the systemic amyloidoses, for instance lysozyme amyloidosis, the presence of literally kilograms of amyloid deposits in multiple organs is the primary cause of disease [136].

Determining the individual microscopic processes involved in the formation of amyloid fibrils is therefore a crucial step in understanding the mechanism by which amyloid formation is associated with pathology in these diseases. Significant advances in our ability to determine aggregation mechanisms have been made through a combination of experiments to probe individual microscopic processes of amyloid formation with integrated rate laws.

Protein aggregation consists of a number of distinct molecular processes. The majority of amyloid forming proteins display a sigmoidal kinetic profile with a lag time, which can be shortened through the addition of pre-formed seed fibrils [123] (Figure 6). This demonstrates that nucleation processes have a major influence on the kinetics of amyloid formation. The pre-formed fibrils act as templates for the addition of further monomer resulting in fibril growth. In the majority of cases, particularly in pathological protein aggregation, primary nucleation (the initial formation of aggregates) and elongation (fibril growth) alone are not sufficient to describe the observed kinetics. The autocatalytic replication of fibrils is a key mechanism by which fibril formation occurs, either through nucleation on the fibril surface, or through the fragmentation of fibrils [137]. Both of these processes increase the number of fibril ends capable of further growth via elongation in an exponential manner. In particular, in many cases experimental samples are subjected to strong mechanical action, for instance shaking at several hundred revolutions per minute, which can induce the fragmentation of fibrils and strongly accelerate the aggregation reaction [123]. The primary nucleation rate is dependent on the concentration of soluble (normally monomeric) protein, the fragmentation rate is dependent on the concentration of fibrillar protein, and the rate of secondary nucleation depends on the concentration of both fibrillar and soluble protein (Figure 6).

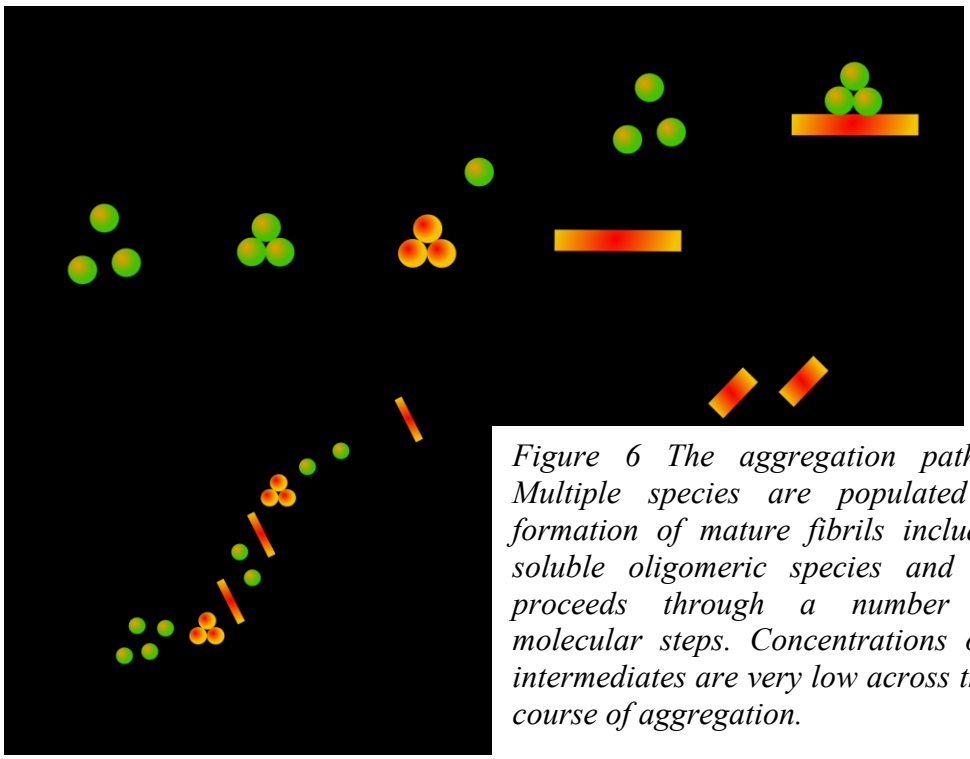

Figure 6 The aggregation pathway of αS. Multiple species are populated during the formation of mature fibrils including multiple soluble oligomeric species and the reaction proceeds through a number of discreet molecular steps. Concentrations of oligomeric intermediates are very low across the entire time course of aggregation.

Analysis of experimental kinetic data, for example from ThT intensity traces, can be performed by global fitting to integrated rate equations for amyloid assembly derived from a model of the underlying molecular processes that make up the aggregation network. The fitted parameters correspond to physical properties of the system such as rate constants of the individual microscopic processes (e.g. elongation, nucleation),

nucleus size, and saturation concentrations. For a particular aggregation reaction, multiple kinetic traces at different reagent concentrations can be analyzed with a single rate law using global fitting allowing the aggregation mechanism to be distinguished. Fitting software which can perform global analysis of kinetic data is available online (www.amylofit.ch.cam.ac.uk/).

Global fitting of kinetic models to single-molecule FRET data [105] at varying initial monomer concentrations revealed a nucleation-conversion-polymerisation model for αS aggregation in which low-FRET oligomers are formed from monomeric protein in solution which then convert through a unimolecular monomer-independent conversion reaction to high-FRET oligomers. These high-FRET oligomers convert through a similar reaction to fibrils, which then elongate by monomer addition. The rate constants for each of these steps were determined, allowing predictions to be made about the seeding efficiency of oligomers and fibrils and their relative abundance under a range of conditions.

Global kinetic analysis of αS aggregation data (monitored by ThT fluorescence) in the presence of lipid vesicles has shown that heterogeneous primary nucleation at the membrane surface occurs several orders of magnitude faster than homogeneous primary nucleation involving free monomers in bulk solution. This heterogeneous nucleation reaction also requires a conversion step to form a growth-competent nucleus, which then elongates by monomer addition [120].

By fitting these mechanistic models to experimental kinetic data and obtaining rate constants for the individual molecular processes that govern fibril assembly, the effect of disease-related mutations [77], solution conditions [138], protein interactions [127], lipid vesicles [126] and small molecules [139] on individual steps in amyloid formation have been determined. Understanding how these factors affect each step is crucial, as the relative nucleation, conversion and elongation rates determine critical parameters such as the abundance of potentially cytotoxic oligomers [105,140].

Recent studies have also deduced the effect of pathological mutations on the microscopy processes involved in protein aggregation. αS mutations have a fairly weak effect on the elongation rate of αS fibrils, but have a strong effect on surface based nucleation processes, either primary nucleation on the surface of lipid membranes, or secondary nucleation on the surface of pre-formed fibrils [77]. Investigations of the earliest stages in the aggregation process by single-molecule methods have revealed two distinct pathways for αS oligomerisation. Wild-type αS, A30P and G51D form oligomers of around 30 monomers, while E46K, H50Q and A53T rapidly form larger fibrillar species containing at least 100 monomers. Strikingly, while co-aggregation is observed between proteins within these two groups, wild-type, A30P and G51D do not co-aggregate with E46K, H50Q or A53T, suggesting that there are parallel pathways for the initial formation of fibrils by these two classes of mutants [111].

d) Structural characterisation of toxic intermediate species

Oligomeric αS species have been identified, isolated and characterized by a variety of biophysical methods [85,100,103,111,130,141]. They are generally composed of tens of monomers, and analytical ultracentrifugation of isolated oligomers revealed two broad subgroups of oligomer sizes, one containing an average of 18 +/- 7 monomers and the other an average of 29 +/- 10 monomers [130]. These species adopt a spherical morphology as observed by AFM and TEM and contain antiparallel β-sheet structure. Since amyloid fibrils are composed of highly ordered parallel β-sheets, rearrangements of β strands from an antiparallel to a parallel configuration have been proposed as a structural basis for the slow conversion step in fibril assembly. Comparison of ANS binding characteristics showed an increased exposed hydrophobic surface area for each monomer for oligomers as compared to fibrils, and this may play a role in their propensity to disrupt lipid membranes [142,143]. This has been further demonstrated by hydrophobicity mapping (sPAINT, Figure 5B) at the single molecule level [118]. 3D reconstructions of cryo-EM images have revealed a cylinder-like structure of isolated oligomeric species, with a low-electron density region running through the cylinder [130].

The observation of particular oligomeric states with increased toxicity, and the ability to detect changes in the number of early oligomers over time are promising steps towards delineating the effect of oligomer structure and concentration on cellular toxicity. Recently, structural characterization of two types of αS oligomers using solution and solid state NMR has provided residue-specific information on their interaction with lipid membranes. These experiments revealed a highly lipophilic region within the oligomers that promotes strong interactions with the membrane surface and a rigid β-sheet rich oligomeric core that inserts into the lipid bilayer and disrupts membrane integrity [144].

However, while high-resolution structures of amyloid fibrils of αS [145,146] have been determined, high-resolution structures of oligomeric intermediates have to date not been obtained due to a combination of their size, their transient nature and the heterogeneity of oligomeric species. Advances in high-resolution cryo-EM, which have allowed near-atomic resolution structure determination of large multi-subunit complexes, have already resulted in greatly improved knowledge of amyloid fibril structure [146,147], and have paved the way for investigations into oligomeric intermediates.

Aside from higher resolution structures, it is important to determine the relative concentrations of oligomeric species *in vivo,* as well as the extent to which the structural features of oligomers formed *in vitro* correspond to those *in vivo*. To this end, improved tools for the detection of oligomers in live cells or human samples are required. In addition to the fundamental biological knowledge this would bring, these have obvious applications in detecting potentially toxic aggregates in patient samples.

Finally, the conversion of αS to a toxic oligomeric form may be influenced by post-translational modifications or truncations, and the major, or most toxic, species *in vivo* may not be composed purely of unmodified αS. How these modifications affect the structural features of oligomers have yet to be investigated in detail.

5) Conclusion

Understanding the mechanistic details of fibril-formation by αS is crucial for the rational design of therapeutic interventions. The exact mechanism of aggregation and cellular toxicity will determine whether inhibiting specific processes, for instance nucleation or the growth of aggregates, will reduce the number of cytotoxic species formed during fibril formation.

In this review, we have outlined techniques to detect and characterise multiple aggregated species of αS, from early oligomeric species to mature fibrils. In addition, we have provided a brief introduction to the global fitting of kinetic data to extract rate constants for underlying molecular processes. Combining these techniques will allow us to predict, and test experimentally, how targeted interventions to aggregation processes will result in changes in the abundance of cytotoxic species formed.

Although much progress has been made, there are still many questions that remain unanswered. Many of the breakthroughs on understanding αS oligomer formation have been made using *in vitro* experimentation, and it will now be important to determine whether these findings are replicated *in cellulo*, and even *in vivo*. The presence of machinery to prevent protein aggregation *in vivo*, in addition to the complex nature of cells, and the existence of many possible nucleation sites is very likely to heavily affect how αS behaves. Additionally, the other proteins found within inclusions may also have an important role in the disease, as well as the many post-translational modifications that are found on these and αS. The extent to which the spreading of pathology is due to templated seeding, or to the spreading of cellular stress, is also still unclear. The number of aggregates required to induce oxidative stress has been estimated to be around 100-fold lower than the number required to effectively seed aggregation at physiological αS concentrations [105]. This suggests that the spreading of aggregation always occurs under conditions of cellular stress, which itself promotes αS aggregate formation, and indeed a number of proteins implicated in the oxidative stress response are found in Lewy bodies. The interplay between αS misfolding and aggregation, oxidative stress and neuroinflammation, and the selective vulnerability of dopaminergic neurons, is complex and remains to be fully delineated.

Overall, it is only through further understanding of these processes that we will be able to generate effective diagnostics and therapeutics for the many synucleinopathies that exist.

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